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Expression Profile of NOTCH3 in Mouse Spermatogonia

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Abstract
Stable and sustainable spermatogenesis is supported by the strict regulation of self-renewal and differentiation of spermatogonial stem cells (SSC), which are a rare population of undifferentiated spermatogonia. It has been revealed that some signaling factors regulate the self-renewal of SSC; however, the molecular mechanism of SSC maintenance is still not completely understood. Notch signaling is an evolutionarily conserved juxtacrine signaling that plays important roles in the cell fate determination of various tissue stem cells. Recently, analyses of loss- and gain-of-function suggested that Notch signaling was necessary for normal spermatogenesis. However, the expression of Notch signal components in spermatogonia is still unclear. Here, we analyzed the distribution of NOTCH3-expressing spermatogonia and the target genes. Double immunostaining with differentiation markers revealed that NOTCH3 was expressed in some undifferentiated and differentiated spermatogonia in mouse testes. To define the target gene of Notch3 signaling in spermatogonia, we analyzed the mRNA expression pattern of *Hes* and *Hey* family genes during testis development. *Hes1* abundance was decreased during testis development, suggesting that spermatogonia may express *Hes1*. Immunohistochemical analysis showed that HES1 was expressed in prepubertal spermatogonia, whereas it was expressed predominantly in adult Sertoli cells and weakly in adult spermatogonia. Furthermore, NOTCH3-HES1 double-positive spermatogonia were in pup and adult testes. These results suggest that Notch3 signaling in spermatogonia could promote *Hes1* expression.

Abbreviations used in this paper

dpp	days postpartum
ICD	intracellular domain
N3ECD	Notch3 extracellular domain
N3ICD	Notch3 intracellular domain
PBS	phosphate-buffered saline
SSC	spermatogonial stem cells
WT1	Wilms' tumor 1

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Introduction

In mouse testes, stemness is maintained in some spermatogonia in the primitive state, which are referred to as undifferentiated spermatogonia (A_{single}, A_{paired}, and $A_{aligned}$) [Boitani et al., 2016]. Molecular signaling pathways and somatic cells (such as Sertoli cells, myoid cells, and Leydig cells) govern spermatogonial stem cell (SSC) differentiation. For example, retinoic acid signaling induces the transition from undifferentiated spermatogonia to differentiated spermatogonia (A_{1-4}, intermediate and type B), committed towards meiosis [Mark et al., 2015]. However, the glial cell line-derived neurotrophic factor is essential for the self-renewal and survival of SSC; it was secreted from Sertoli cells and peritubular myoid cells [Meng et al., 2000; Chen et al., 2014]. Moreover, the self-renewal of SSC is promoted by fibroblast growth factor 2 from Sertoli cells and colony-stimulating factor 1 from Leydig cells and macrophages [Oatley et al., 2009; Ishii et al., 2012; DeFalco et al., 2015]. SSC maintenance is also controlled by intrinsic factors, including PLZF, DMRT1, NANOS2, and ID4 [Costoya et al., 2004; Sada et al., 2009; Oatley et al., 2011; Zhang et al., 2016]. However, the molecular mechanism of SSC maintenance is not yet completely understood.

Notch signaling is an evolutionarily conserved juxtacrine signaling that plays an important role in the differentiation, proliferation, and maintenance of various tissue stem cells [Koch et al., 2013]. Mammals have 4 Notch transmembrane receptors (NOTCH1–4) and 5 ligands (Jagged1/2 and Dll1/3/4). When the extracellular domain of the Notch receptor interacts with the ligand of neighboring cells, the intracellular domain (ICD) of the Notch receptor is cleaved by γ-secretase. The released Notch ICD translocates into the nucleus and forms a complex with DNA-binding transcription factor RBP-jκ and other coregulators. This complex induces the expression of specific target genes, such as *Hairy/Enhancer-of-split* (*Hes*) and *Hes-related with YRPW* (*Hey*) family genes, which are members of basic helix-loop-helix transcriptional repressors [Iso et al., 2003]. In mouse embryos, HES1, HES5, HEY1, and HEY2 suppress the transcription of the neuronal basic helix-loop-helix genes *Mash1* and *Math*, and they promote the maintenance of neural stem cells [Ohtsuka et al., 1999; Sakamoto et al., 2003; Kageyama et al., 2005].

It has been previously reported that Notch signaling promotes proliferation and inhibits differentiation of the germline stem cells in *Caenorhabditis elegans* [Kimble and Crittenden, 2007]. In mouse testes, Notch signaling inhibition in all testicular cells (by injection of a γ-secretase inhibitor into the seminiferous tubule) induced the collapse of the spermatogenic cycle and generated abnormal spermatozoa [Murta et al., 2014]. However, inactivation of pan-Notch signaling in germ cells by inhibition of the Notch modification enzyme showed normal spermatogenesis [Hasegawa et al., 2012], whereas activation of NOTCH1 in germ cells led to apoptosis of germ cells and abnormal spermatogenesis [Huang et al., 2013]. Thus, the role of Notch signaling in mammalian spermatogenesis is controversial.

Recently, we demonstrated that Jagged1 mRNA and protein were expressed in mouse Sertoli cells [Okada et al., 2016]. As a result, we hypothesize that JAGGED1 of Sertoli cells activates Notch signaling in germ cells. The testicular localization of Notch receptors in mouse testes has been reported in several studies [Dirami et al., 2001; Mori et al., 2003; von Schonfeldt et al., 2004; Hasegawa et al., 2012; Murta et al., 2013], but these reports are not consistent for NOTCH1/2 localization. In contrast, these reports were in accordance with NOTCH3 localization in spermatogonia [Dirami et al., 2001; Mori et al., 2003; Murta et al., 2013]. However, the differentiation state of a NOTCH3-expressing cell in testes is unclear. In this study, we investigated the expression of NOTCH3 and the target genes in developing testes.

Materials and Methods

Animals

Male ICR and WBB6F1-W/W^V (W/W^V) mice were purchased from Japan SLC (Shizuoka, Japan). They were housed in our animal facility on a 12-h light/dark cycle, and they were given access to food and sterilized water ad libitum. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committees of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

Immunohistochemistry

Testes were fixed with 4% paraformaldehyde, frozen in an optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), and cut into 6-μm-thick sections in a cryostat. For HES1 and Wilms' tumor 1 (WT1) staining, nonfixed cryosections were used and fixed with 4% paraformaldehyde for 5 min at room temperature just before blocking. Cryosections were blocked with 5% normal donkey serum in phosphate-buffered saline (PBS) for 1 h at room temperature, and then they were incubated with primary antibodies diluted in 3% bovine serum albumin and 0.1% NaN_3 in PBS at 4°C overnight. Subsequently, sections were washed with PBS and then incubated with secondary antibodies for 1 h at room temperature. Sections were counterstained with 10 μg/mL DAPI, and mounted with 50% glycerol in PBS. Images were assessed with

an Axioplan 2 imaging microscope with AxioVision software (Carl Zeiss, Göttingen, Germany). Notch3 ICD (N3ICD) antibody (sc-7424, 1:100; Santa Cruz, CA, USA), WT1 antibody (sc-192, 1:100; Santa Cruz), TRA98 antibody (73-003, 1:5,000; Bio Academia, Osaka, Japan), Hes1 antibody (sc-25392, 1:100; Santa Cruz), E-cadherin antibody (M108, 1:100; Takara, Shiga, Japan), and KIT antibody (14-1171, 1:400; eBioscience) were used as primary antibodies, and anti-goat IgG Alexa Fluor 488, anti-rabbit IgG Alexa Fluor 594, anti-rat IgG Alexa Fluor 594, and anti-rat IgG Alexa Fluor 488 (all from Thermo Fisher Scientific) were used as secondary antibodies.

qRT-PCR Analysis

Total RNA was extracted from testes of mice aged 7.5, 14.5, 21.5, and 28.5 days postpartum (dpp) and 2-month-old (adult) ICR mice and 2-month-old W/W^V mice using TRIzol reagent (Thermo Fisher Scientific). One microgram of total RNA was reverse transcribed using AMV Reverse Transcriptase XL (Takara) with Oligo dT primer (Invitrogen). qRT-PCR analysis was performed in duplicate using the gene-specific primers with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) employing a StepOnePlus realtime PCR system (Thermo Fisher Scientific). PCR conditions were: 95°C for 5 min, followed by 40–50 cycles of 95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s. The annealing temperatures and primer sequences are listed in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000481772). Transcript levels were normalized to those of *Cyclophilin* expression.

Statistical Analysis

Each experiment was repeated at least 3 times. The ratios of undifferentiated and differentiated spermatogonia expressing NOTCH3 and HES1 were determined by counting all E-cadherin+ or KIT+ spermatogonia, in over 30 seminiferous tubule sections per testis, in all 3 mice at each developmental stage. A 2-tailed Student's *t* test for 2 groups and ANOVA followed by the Tukey's test for multiple group comparisons were used to analyze the significance of differences. $p < 0.05$ was considered statistically significant.

Results

NOTCH3 Was Expressed in Some Spermatogonia in Developing and Adult Testes

First, we investigated the testicular localization of NOTCH3 during the postnatal development of mouse testes via double immunostaining of N3ICD and WT1, which is a Sertoli cell marker [Pelletier et al., 1991]. In 7.5-dpp, 20-dpp, and adult testes, NOTCH3-expressing cells were WT1-negative, they were localized on the basement membrane, and they had an oval nuclear shape (arrows in Fig. 1a–c; isotype control in online suppl. Fig. S1A). Moreover, NOTCH3-expressing cells were costained with TRA98, which is a germ cell marker antibody [Tanaka et al., 1997] (Fig. 1e, f). In testes of W/W^V mutant mice that lacked germ cells [Handel and Eppig, 1979], NOTCH3-expressing cells were not detected (Fig. 1d). The specificity of the Notch3 antibody is presented in online supplementary Figure S1B. Therefore, testicular localization and morphological features revealed that NOTCH3-expressing cells were spermatogonia.

NOTCH3 Was Expressed in Both Undifferentiated and Differentiated Spermatogonia

To reveal the differentiation state of NOTCH3-positive spermatogonia, we used differentiation markers of spermatogonia. E-cadherin and PLZF are expressed in undifferentiated spermatogonia [Costoya et al., 2004; Tokuda et al., 2007]; KIT is expressed in differentiated spermatogonia [Schrans-Stassen et al., 1999]; STRA8 is weakly expressed in differentiated spermatogonia and strongly expressed in preleptotene and early leptotene spermatocytes [Zhou et al., 2008]. Immunostaining analysis revealed that NOTCH3 was expressed in some of E-cadherin-positive undifferentiated spermatogonia in 7.5-dpp and adult testes (arrows in Fig. 2a, b), and the percentage of NOTCH3-expressing cells in undifferentiated spermatogonia was more than approximately 60% for 7.5-dpp and adult testes (Fig. 2e). Similarly, in adult testes, NOTCH3 and PLZF double-positive cells were detected (online suppl. Fig. S2A). However, NOTCH3 was also expressed in some KIT-positive differentiated spermatogonia in 7.5-dpp and adult testes (Fig. 2c, d). In 7.5-dpp testes, KIT-expressing cells were partially merged to E-cadherin-positive cells (online suppl. Fig. S2B). The percentage of NOTCH3-expressing cells in differentiated spermatogonia was significantly decreased in adult testes (Fig. 2f). The majority of NOTCH3-expressing cells were STRA8-negative, but some were weakly STRA8-positive in adult testes (online suppl. Fig. S2C). Furthermore, three fourths of the NOTCH3-expressing spermatogonia were in an undifferentiated state at 7.5 dpp, but the rate was about half in adult testes (Fig. 2g). Thus, heterogeneity in the differentiation state of NOTCH3-expressing spermatogonia was found. Moreover, the signal of N3ICD was detected in the nucleus of spermatogonia (inset in Fig. 2a, b). This nuclear localization of N3ICD suggests the activation of Notch3 signaling in spermatogonia.

Hes1 Abundance Was Decreased in Testis Development

The ICD of Notch receptors (except Notch3) were observed in spermatocytes and spermatids [Mori et al., 2003]; thus, many target genes would be expressed in mouse testes. To find the spermatogonia-specific target

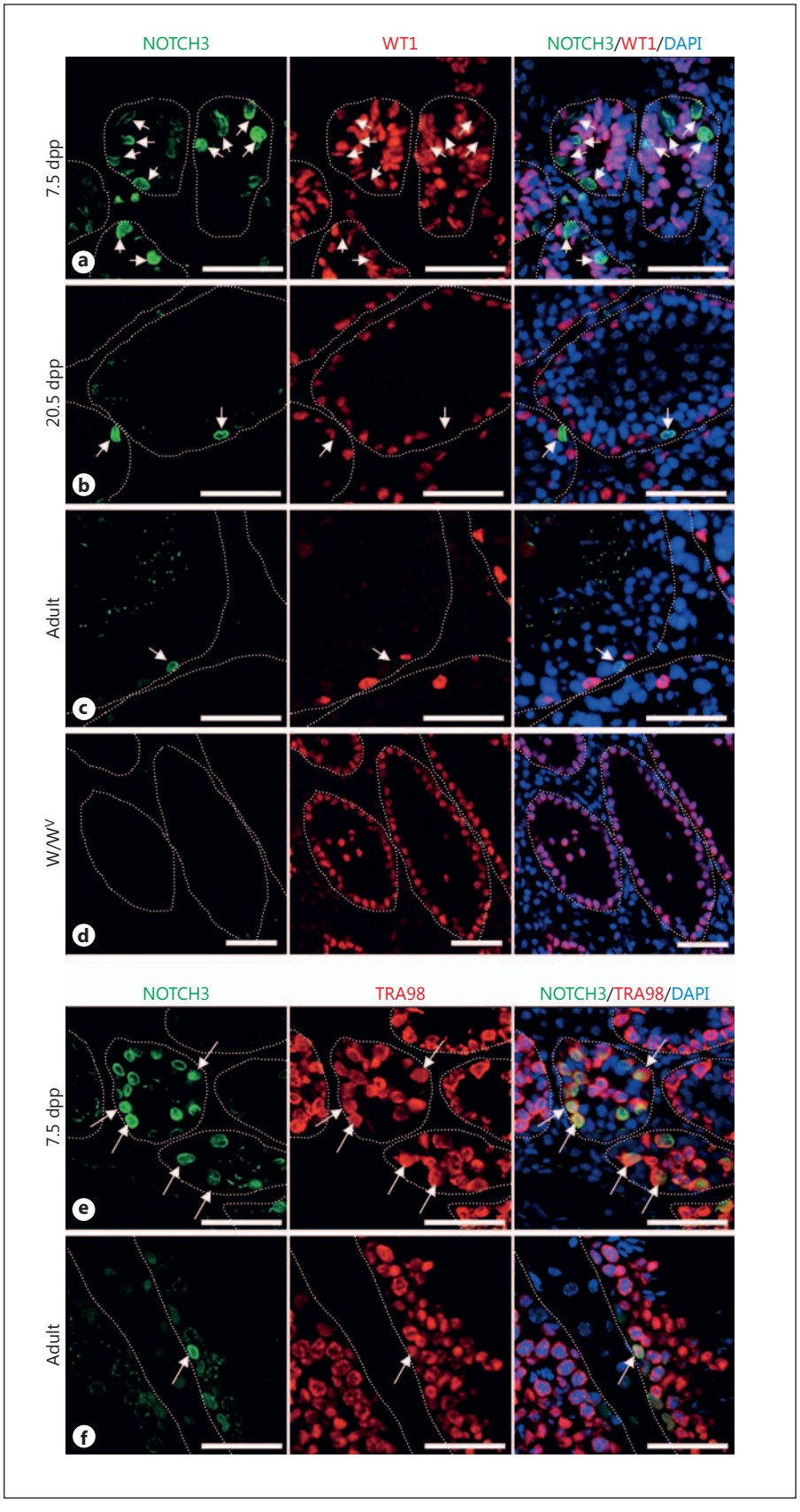

Fig. 1. Double immunostaining with NOTCH3 (green) and Wilms' tumor 1 (WT1) (red) for testes of mice aged 7.5 (**a**) and 20.5 days postpartum (dpp) (**b**) and 2 months (adult; **c**) and W/W^V (**d**) mouse testes. WT1 is a Sertoli cell marker. In postnatal developing testes, NOTCH3-positive cells (arrows) were located on the basement membrane (dotted line) and they were negative for WT1. In W/W^V testes, NOTCH3-positive cells were not detected (**d**). **e**, **f** Double immunostaining with NOTCH3 (green) and TRA98 (red) for 7.5-dpp (**e**) and 2-month-old (**f**) testes. TRA98 was detected in the nuclei of spermatogonia to round spermatids. NOTCH3-positive cells (arrows) were located on the basement membrane (dotted lines) and they were positive for TRA98. DAPI (blue) indicates the nuclei of all testicular cells. Scale bar, 50 μm.

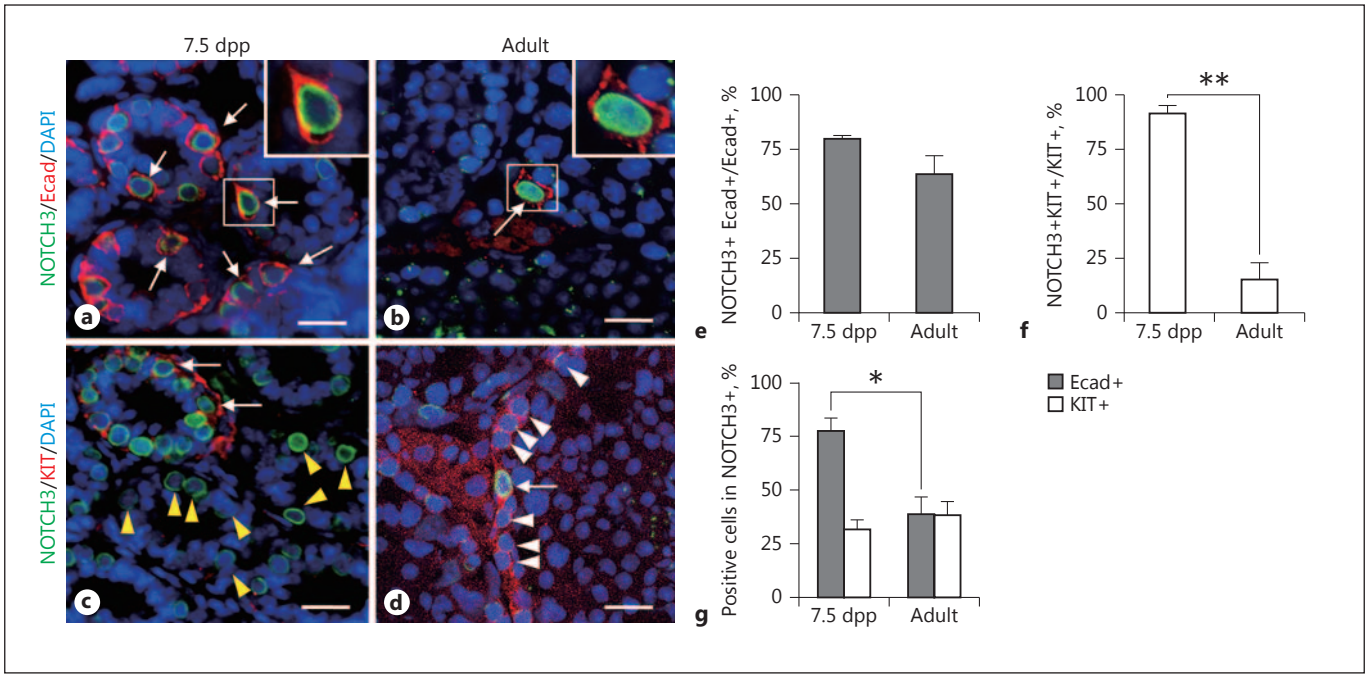

Fig. 2. Double immunostaining with NOTCH3 (green) and E-cadherin (Ecad; red) for testes of mice aged 7.5 days postpartum (dpp) (**a**) and 2 months (**b**). Ecad is expressed in undifferentiated spermatogonia. Arrows indicate NOTCH3 and Ecad double-positive cells. **c**, **d** Double immunostaining with NOTCH3 (green) and KIT (red) for 7.5-dpp (**c**) and 2-month-old (**d**) testes. KIT is expressed in differentiated spermatogonia. Arrows indicate NOTCH3 and KIT double-positive cells. Yellow arrowheads indicate NOTCH3-positive, KIT-negative cells. White arrowheads show NOTCH3-negative, KIT-positive cells. Scale bar, 20 μm. **e** The percentage of NOTCH3-positive cells in Ecad-positive undifferentiated spermatogonia was plotted (n = 3). **f** The percentage of NOTCH3-positive cells in KIT-positive differentiated spermatogonia was plotted (n = 3). **g** The percentage of Ecad-positive and KIT-positive cells in NOTCH3-positive cells was plotted (n = 3). Error bars represent the standard error of the mean (SEM). Asterisks indicate significant differences by the Student's t test (* $p < 0.05$, ** $p < 0.01$).

gene, the expression pattern of *Hes* and *Hey* family genes were analyzed. Hematoxylin and eosin staining demonstrated the cell composition of postnatal testis development (online suppl. Fig. S3). In a later stage of testis development, pachytene spermatocytes (14.5 dpp), round spermatids (21.5–28.5 dpp), and elongating spermatids (adult) sequentially appeared and occupied the seminiferous tubule, and as a result the populations of spermatogonia and Sertoli cells were gradually decreased. Therefore, the spermatogonia-specific gene abundance should be decreased along with testicular development in the whole testis sample. qRT-PCR using whole testes revealed that the expression patterns of *Hes* and *Hey* family genes could be categorized into 4 patterns: (1) a gradually decreasing pattern (*Hes1*), (2) a radically increasing pattern (*Hes2*), (3) a gradually increasing pattern (*Hey1* and *Hey2*), and (4) constant expression (*Hes5*) (Fig. 3). This result suggested that *Hes1* could be the target gene of Notch3 signaling in spermatogonia. Moreover, reanalysis of published microarray data (GEO accession No. GSE4193) [Namekawa et al., 2006] showed that *Hes1* expression was higher in spermatogonia than in pachytene spermatocytes and round spermatids (online suppl. Fig. S4A). In order to eliminate the possibility that the decreasing pattern of *Hes1* expression was derived from Sertoli cells, mature and immature Sertoli cells were analyzed (online suppl. Fig. S4B). *Hes1* expression in immature Sertoli cells was lower than that in mature Sertoli cells, indicating that *Hes1* was expressed in spermatogonia during the early stage. Moreover, other *Hes* and *Hey* family genes in Sertoli cells were weakly expressed relative to whole testes of 7.5-dpp mice.

HES1 Protein Was Expressed in Prepubertal/Adult Spermatogonia and Adult Sertoli Cells

To confirm whether HES1 is expressed in spermatogonia, we analyzed the testicular localization of HES1 protein in mouse testes by double immunostaining with TRA98. HES1 was found to be expressed in spermatogonia and a small portion of Leydig cells but not in Sertoli

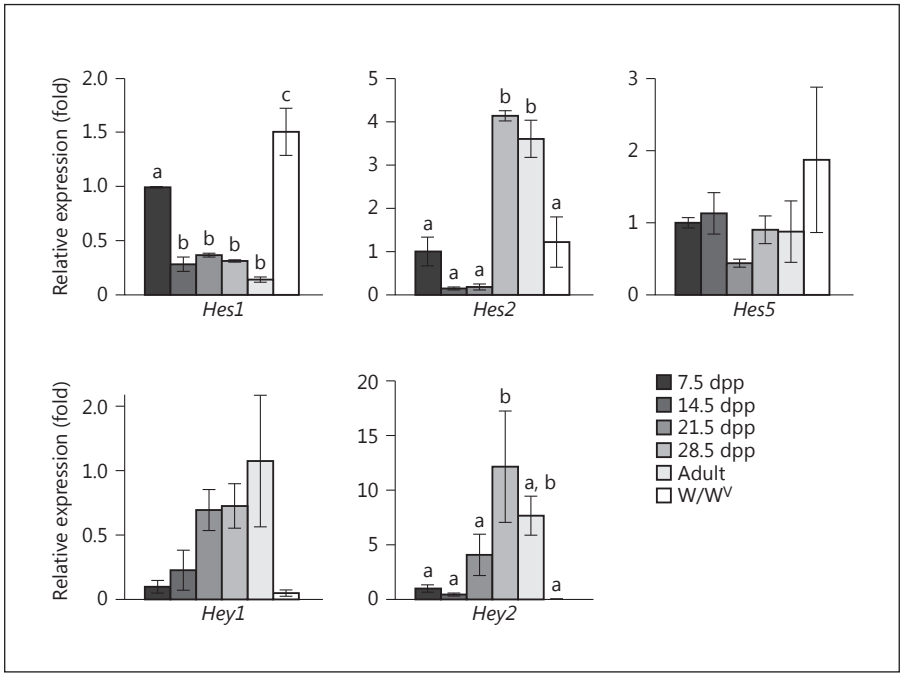

Fig. 3. qRT-PCR analysis of *Hes* and *Hey* family gene expression during the postnatal development of testes. Total RNA was harvested from whole testes in mice aged 7.5, 14.5, 21.5, and 28.5 days postpartum (dpp) and 2 months and W/W^V mice (n = 3). The gene expression level was normalized to the housekeeping gene *Cyclophilin*, and the fold change compared to 7.5 dpp was plotted. Error bars represent SEM. Different letters indicate significant differences between groups by the Tukey's test ($p < 0.05$).

cells of 7.5-dpp testes (Fig. 4a). In adult testes, HES1 was expressed in Sertoli cells and some spermatogonia, but at some testicular stages weak signals were also detected in spermacytes and spermatids (Fig. 4b; isotype control in online suppl. Fig. S1C).

HES1 Was Expressed in Some NOTCH3-Positive Spermatogonia

NOTCH3 and HES1 coexpressing cells were detected in 7.5-dpp and adult testes (Fig. 5a, b). This result suggests that Notch signaling was activated in these cells. However, the percentage of HES1-expressing cells in NOTCH3-positive spermatogonia was around 20–22% for 7.5-dpp and adult testes (Fig. 5c).

HES1 Was Expressed in Undifferentiated and Differentiated Spermatogonia

NOTCH3 and HES1 coexpressing cells exhibited morphological features of type A spermatogonia (A_{single} – A_4), with a flattened cell shape and ovoid nuclei (online suppl. Fig. S5). Next, we investigated the differentiation state of HES1-expressing spermatogonia by differentiation markers of spermatogonia. Immunostaining of HES1 and E-cadherin revealed that HES1 was expressed in some E-cadherin-positive undifferentiated spermatogonia in 7.5-dpp and adult testes (Fig. 6a, b). The percentage of HES1-positive cells in undifferentiated spermatogonia was significantly increased in adult testes compared to 7.5-dpp testes (Fig. 6e). In contrast, KIT-positive differentiated spermatogonia partially expressed HES1 in 7.5-dpp testes but almost not in adult testes (Fig. 6c, d, f).

Discussion

We showed that NOTCH3 was expressed in both E-cadherin-positive undifferentiated and KIT-positive differentiated spermatogonia in 7.5-dpp and adult mouse testes (Fig. 2). Previous investigations had reported that spermatogonia expressed NOTCH3 in mouse testes [Dirami et al., 2001; Mori et al., 2003; Murta et al., 2013], but the differentiation state of NOTCH3-expressing spermatogonia had not yet been analyzed. Our work is the first to reveal the differentiation state of NOTCH3-expressing spermatogonia. Over half of the E-cadherin-positive undifferentiated spermatogonia expressed NOTCH3, suggesting that Notch3 signaling may be involved in differentiation and proliferation of undifferentiated spermatogonia (Fig. 2). To investigate the role of Notch3 signaling in spermatogonia, we tried to detect and isolate NOTCH3-expressing spermatogonia by fluorescence-activated cell sorting using a useful anti-Notch3 extracellular domain (N3ECD) antibody (12-5763; eBio-

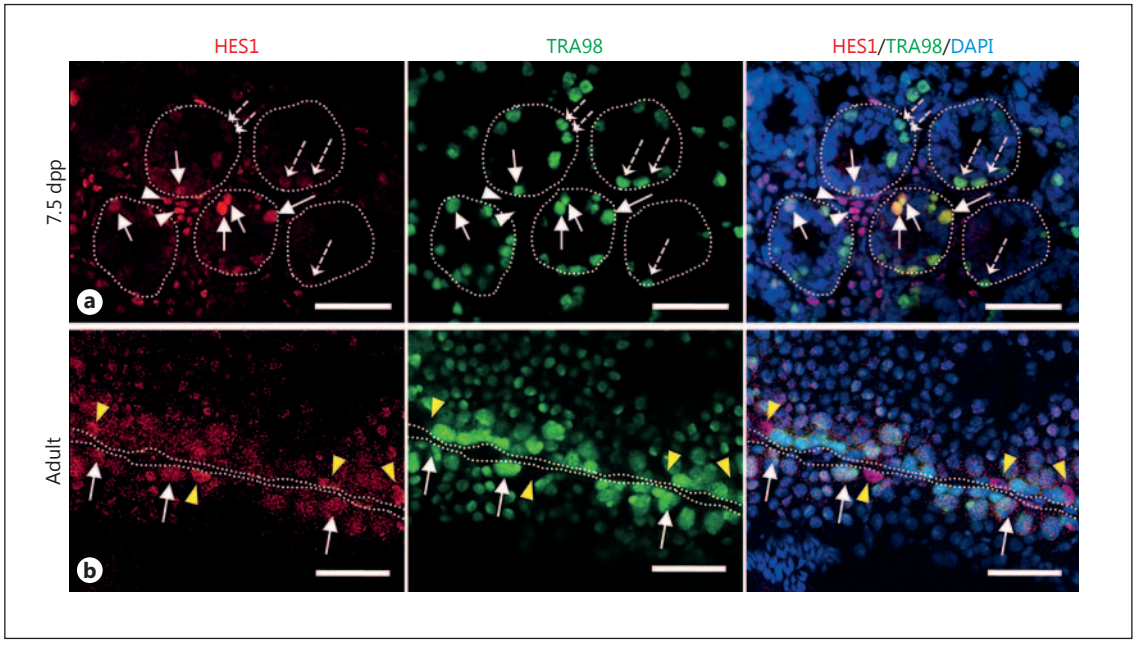

Fig. 4. Double immunostaining with HES1 (red) and TRA98 (green) for testes of mice ages 7.5 days postpartum (dpp) (**a**) and 2 months (**b**). TRA98 is a germ cell marker. HES1-positive germ cells (HES1+/TRA98+; arrows) were localized on the basement membrane (dotted line) in 7.5-dpp and 2-month-old testes. Broken arrows indicate HES1-negative, TRA98-positive cells. HES1-expressing Leydig cells in 7.5-dpp testes (white arrowheads) and HES1-expressing Sertoli cells in adult testes (HES1+/TRA98–; yellow arrowheads) were also presented. Scale bar, 50 μm.

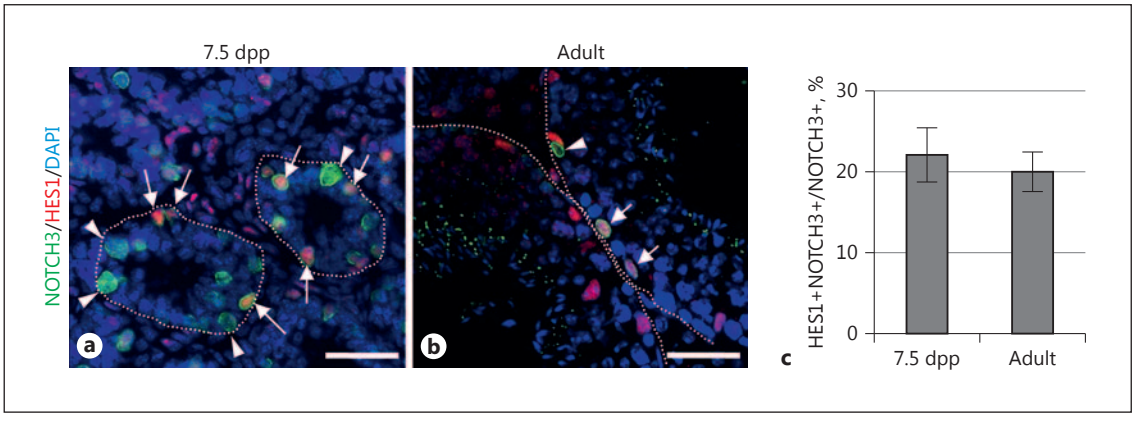

Fig. 5. Double immunostaining with NOTCH3 (green) and HES1 (red) for testes of mice aged 7.5 days postpartum (dpp) (**a**) and 2 months (**b**). While NOTCH3 and HES1 double-positive cells (arrows) were detected in 7.5-dpp and 2-month-old testes, NOTCH3-positive HES1-negative cells (arrowheads) are also presented. Dotted lines represent the basement membrane. Scale bar, 20 μm. **c** The ratio of NOCTH3 and HES1 double-positive cells in NOTCH3-expressing spermatogonia was plotted (n = 3). Error bars represent the SEM.

science), but we could not (data not shown). Notch signaling activation cleaves NECD by metalloprotease ADAM 10/17 [Koch et al., 2013], and the nuclear localization of N3ICD indicated the activation of Notch3 signaling (Fig. 1, 2, 5). Therefore, N3ECD may be cleaved in Notch3-expressing spermatogonia.

Notch3 knockout mice exhibited an abnormal artery morphology and a decreasing thymocyte number, where-

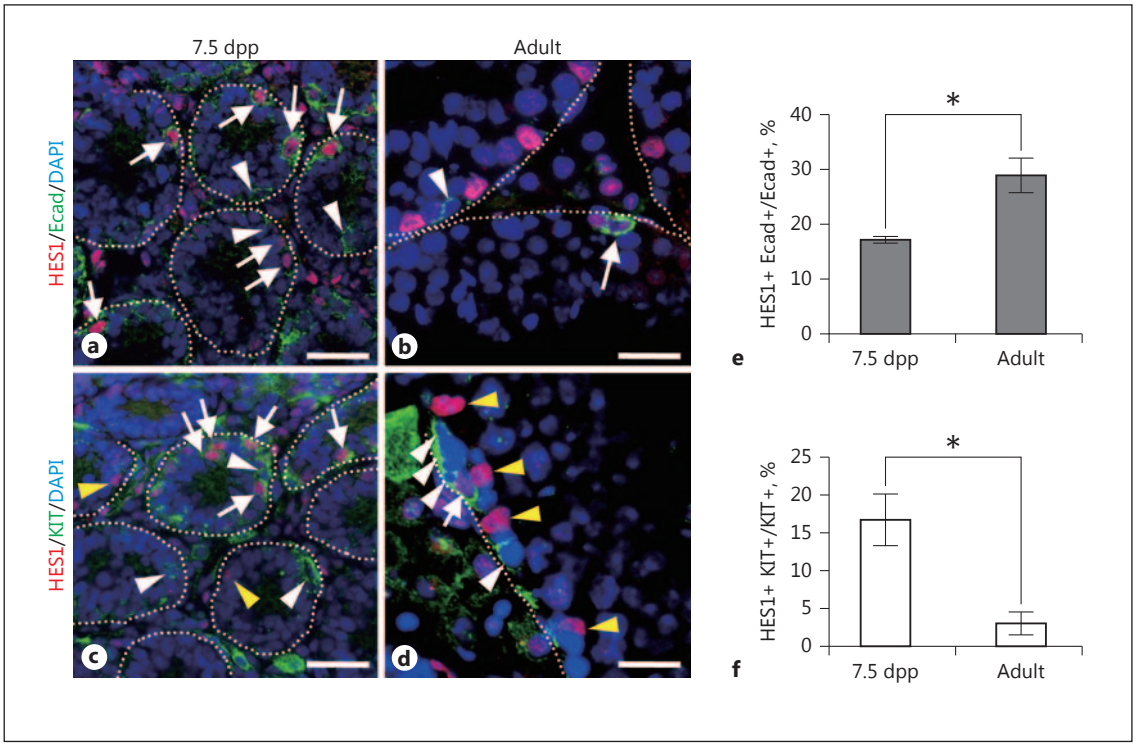

Fig. 6. Double immunostaining with HES1 (red) and E-cadherin (Ecad; green) for the testes of mice aged 7.5 days postpartum (dpp) (**a**) and 2 months (**b**). Arrows indicate HES1 and Ecad double-positive cells. Arrowheads indicate HES1-negative, Ecad-positive cells. **c**, **d** Double immunostaining with HES1 (red) and KIT (green) for 7.5-dpp (**c**) and 2-month-old (**d**) testes. Arrows indicate HES1 and KIT double-positive cells. Yellow arrowheads indicate HES1-positive, KIT-negative cells. White arrowheads show HES1-negative, KIT-positive cells. Scale bars, 20 μm. **e** The percentage of HES1-positive cells in Ecad-positive undifferentiated spermatogonia was plotted ($n = 3$). **f** The percentage of HES1-positive cells in KIT-positive differentiated spermatogonia was plotted ($n = 3$). Error bars represent the SEM. Asterisks indicate significant differences by the Student's t test (* $p < 0.05$).

as there was no effect on fertility [Krebs et al., 2003; Domenga et al., 2004; Kitamoto et al., 2005]. Previous reports have shown that NOTCH1/2 are expressed in spermatogonia, spermatocytes, and spermatids [Mori et al., 2003; Murta et al., 2013], suggesting that the function of Notch3 is redundant in Notch3 mutant spermatogonia.

The decreasing pattern of *Hes1* expression during the development of testes suggested that *Hes1* was expressed in spermatogonia. Meanwhile, expression analyses of Hes2 and Hey1/2 (Fig. 3; online suppl. Fig. S4A and online suppl. Fig. S4B) indicated their constant and ubiquitous expression in germ cells. Our localization analysis revealed that HES1 was expressed in spermatogonia at 7.5 dpp as well as in Sertoli cells and some spermatogonia in adulthood. HES1 expression in adult Sertoli cells was confirmed by W/W^V testes (Fig. 3). HES1 localization in adult testes was in agreement with another report [Murta et al., 2013]. Hasegawa et al. [2012] only found HES1 in Sertoli cells of adult mice. This inconsistency may have resulted from a weak expression level of HES1 in spermatogonia compared to Sertoli cells (Fig. 4–6).

We focused on Notch3-Hes1 signaling in this study; nuclear localization of N3ICD suggested Notch3 signaling activation in NOTCH3-expressing spermatogonia (Fig. 2a, b). Indeed, we found NOTCH3 and HES1 double-positive spermatogonia (Fig. 5). However, NOTCH3-positive, HES1-negative spermatogonia were also present in 7.5-dpp and adult testes. These results indicate the activation pathway of Notch3 signaling via other *Hes/Hey* family genes. Murta et al. [2013] reported that Hes5 was expressed in Sertoli cells and elongated spermatids; such target genes would be correlated.

Testicular localization analysis revealed that HES1 was expressed in both E-cadherin-positive undifferentiated and KIT-positive differentiated spermatogonia at 7.5 dpp, but it was almost not expressed in KIT-positive dif-

ferentiated spermatogonia in adult testes (Fig. 6e, f; online suppl. Fig. S6). Recently, Okuda et al. [2015] reported that NKAPL, which is a suppressor of Notch signaling, was expressed in differentiated spermatogonia in adult testes. It was suggested that such a regulating factor could suppress HES1 expression in NOTCH3-expressing differentiated spermatogonia.

Furthermore, the microarray data by Ikami et al. [2015] showed that *Hes1* was highly expressed in GFRα1-positive undifferentiated spermatogonia of adult testes but weakly expressed in NGN3-positive differentiating or KIT-positive differentiated spermatogonia. Previously, Huang et al. [2013] indicated that the *Hes* family gene suppressed *Ngn3* expression in spermatogonia. These results suggest that HES1 expressed in NOTCH3-positive cells might maintain the undifferentiated state of adult spermatogonia.

Overall, our study revealed that NOTCH3 and HES1 were expressed in undifferentiated and differentiated spermatogonia. Notch3-Hes1 signaling could contribute to proliferation and differentiation of spermatogonia. To reveal the role of Notch3 and Hes1 in spermatogonia, further functional analysis is necessary.

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Disclosure Statement

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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